Facilitated transport of 3-*O*-methyl-D-glucose in human polymorphonuclear leukocytes

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Transport of the nonmetabolizable hexose analogue 3-*O*-methyl-D-glucose (3OMG) was measured in human polymorphonuclear leukocytes at 37°C, pH 7.4. 3OMG at very low concentration (0.05 mM) equilibrated with the intracellular water with a rate constant of about 0.08 s^{-1}. Transport of 3OMG in the presence of 20 μM cytochalasin B and transport of L-glucose were insignificant. Countertransport of ^{14}C-labelled 3OMG was demonstrated. Exchange of 3OMG between the extracellular and intracellular water showed saturation with a K_m of about 4 mM. Thus, the transport of 3OMG is mediated almost exclusively by facilitated diffusion.

Glucose transport *3-O-Methylglucose* *Polymorphonuclear leukocyte*

1. INTRODUCTION

The PMNL depends on glucose as a source of energy for motility, chemotaxis, phagocytosis and bactericidal activity. Early experiments were interpreted to show that D-glucose and 3OMG were taken up in these cells by 'free' (nonmediated) diffusion, i.e. not facilitated by a specific transporter [1]. However, subsequent studies demonstrated that uptake of labelled 2-deoxy-D-glucose, which is phosphorylated but not further metabolized, is inhibited by D-glucose (not not L-glucose) and by cytochalasin B [2]. Moreover, uptake of 2-deoxyglucose (30 min at 37°C) showed saturation with an apparent K_m of 0.5 mM [3]. Together, these results provided indirect evidence for facilitated diffusion of 2-deoxyglucose.

3OMG has been reported to be taken up in human PMNLs by nonmediated diffusion and not by a specific transporter [1,4]. We decided to reinvestigate transport of 3OMG for the following reason. If the affinity of 3OMG to the PMNL transporter is so low that uptake occurs essentially via nonmediated diffusion, then this transporter would be markedly different in sugar specificity from those characterized in other cell types, e.g. adipocytes [5], hepatocytes [6] and lymphocytes [7]. If, on the other hand, 3OMG is taken up by facilitated diffusion, then transport can be measured directly in PMNL. This is important because uptake of 2-deoxyglucose will always be determined by the combined activity of the transport system and the hexokinase. Therefore, it is not possible to identify the rate-determining step from the steady-state 2-deoxyglucose uptake rates as measured in previous studies [2–4].

Abbreviations: PMNL, polymorphonuclear leukocyte; 3OMG, 3-*O*-methyl-D-glucose

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2. MATERIALS AND METHODS

3-*O*-[^{14}C]Methyl-D-glucose (53 Ci/mol) and ^3H_2O (0.3 mCi/mol) were purchased from Amersham. L-[1-^3H]Glucose (18 Ci/mmol) was from New England Nuclear. Unlabelled 3OMG was

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from Sigma, cytochalasin B from Aldrich, phloretin from K & K Laboratories and dextran T 110 and Percoll from Pharmacia. Other reagents were analytical grade.

PMNLs were prepared from normal blood donors in the following way. 450 ml fresh blood was mixed with 63 ml of 0.9% NaCl containing 90 mM citrate and 2 mM adenine, followed by centrifugation for 5 min at $6000 \times g$ and about 20 ml of the layer above the packed erythrocytes ('buffy coat') was recovered. 1 vol. of 10% dextran (in 0.9% NaCl containing 20 000 U heparin) was added to 9 vols of buffy coat to increase rouleaux formation of the remaining erythrocytes. After sedimentation for 30 min at 37°C the supernatant, containing PMNLs, mononuclear cells, thrombocytes and still some erythrocytes, was centrifuged for 4 min at about $150 \times g$, followed by resuspension of the cells in buffer containing 31 mM Mops and Krebs' salts as described [8], centrifuged again and resuspended to a volume of 2.5 ml. The cells were layered on top of a preformed linear Percoll gradient adjusted to 1.044–1.097 kg/l and centrifuged for 10 min at about $4000 \times g$. The erythrocytes were stuck to the bottom of the tube after this procedure and the PMNLs were recovered from the band of highest density near the bottom. They were then washed twice in the Mops buffer and adjusted to a concentration of about 1×10^8 cells/ml.

The preparation contained 95–99% PMNLs and 1–5% lymphocytes. The yield was 40% of the PMNLs present in the buffy coat and about 90% of the PMNLs present in the supernatant after rouleaux formation. The cells did not clump or adhere to the wall of the tube. More than 99% of the cells excluded trypan blue. Other experiments were performed to confirm the metabolic viability of the cells. Thus, the rates of glycogen synthesis per PMNL from 1–5 mM ^{14}C-labelled glucose were closely similar in the present purified preparation and in a previously described [8] less pure preparation (i.e. without gradient centrifugation).

Transport experiments were carried out at 37°C, pH 7.4, essentially as described for cultured human lymphocytes [7]. In brief, 15 μl buffer with 56 nCi (10^5 cpm) labelled and, as required, unlabelled 3OMG was placed in a 4.5 ml roundbottom mini scintillation vial (Hansac Plastic, Hasselager, Denmark). PMNLs (about 1×10^8/ml) were allowed to equilibrate with unlabelled 3OMG for 15 min at 37°C. At time zero, 45 μl of the PMNL suspension was squirted on to the 15 μl buffer containing isotope and unlabelled 3OMG. Thus, the 3OMG concentration was the same on both sides of the membrane and the equilibration of the extracellular [^{14}C]3OMG with its intracellular distribution space was measured (equilibrium exchange). Timing was carried out using a metronome. Incubations were terminated by the addition of 3.5 ml stopping solution (0.3 mM phloretin in Mops buffer containing 0.1 μM $HgCl_2$) as described [7]. The cells were pelleted by centrifugation at about $4000 \times g$ for 1 min, the supernatant was discarded and the procedure was repeated once. Finally, 2.5 ml of scintillation fluid was added and radioactivity determined. Blank values, determined by the addition of stopping solution before the cells, were subtracted from all measurements and they contained 8–10% of the counts present in the pellet when the cells were allowed to equilibrate with labelled 3OMG for 3 min.

In some experiments the cell pellet was resuspended in 1 ml H_2O and boiled for 5 min. The denatured protein was removed by centrifugation and the supernatant was applied to a 0.7×4 cm anion-exchange column (Bio-Rad AG 1-X8) as described in [9]. ^{14}C-labelled 3OMG was eluted by washing with 2.5 ml of 1 mM 3OMG. Phosphorylated 3OMG was initially retained on the column and was subsequently removed by washing with 12.5 ml of 0.2 M formic acid in 0.5 M ammonium acetate.

Distribution spaces for ^3H_2O, labelled 3OMG and labelled L-glucose were determined by transferring 100 μl suspension of the incubated cells (without wash) to 500 μl microfuge tubes containing 100 μl dibutylphthalate/dinonylphthalate (3 : 1, d 1.0245) followed by centrifugation. The tube was cut and radioactivity in the pellet and supernatant fractions were measured.

All experiments were carried out at least 4 times with essentially the same results.

3. RESULTS

Table 1 shows an intracellular ^3H_2O distribution space (^3H_2O space minus L-glucose space) of about 26 μl in 1×10^8 cells. This is in broad agreement with a mean cell volume of 36×10^{-8} μl calculated

Table 1

Distribution space for ^3H_2O and [^{14}C]3OMG in PMNLs

	Distribution space (μl/10^8 cells)				
	No wash			Wash	
	^3H_2O	3OMG	L-Glucose	3OMG	L-Glucose
Total cell pellet	32.0±3.2	25.4±1.8	5.9±0.3	17.5±1.1	1.2±0.2
Intracellular	26.1±3.3	19.5±1.9	–	16.3±1.2	–

The cells (1×10^8/ml) were incubated for 2 min with 6×10^5 cpm/ml 3-*O*-[^{14}C]methyl-D-glucose or ^3H_2O. L-[1-^{3}H]Glucose was added immediately before termination of the incubations. Distribution spaces without wash or after wash with stopping solution were determined as described in section 2. The intracellular distribution spaces for 3OMG and ^3H_2O without wash are calculated as the total spaces minus the L-glucose space. The intracellular distribution space for 3OMG after wash is calculated as its total space minus a blank value of 1.2 μl/10^8 cells. This is indistinguishable from the total L-glucose space after wash. The results are mean values of 3 replicates ± 1 SD

from a mean diameter of 8.8 μm (with very little scatter) as measured under the microscope. The intracellular distribution space for 3OMG was consistently 20–30% lower than the ^3H_2O space measured in the same way. Thus, 3OMG is restricted from a minor part of the intracellular water, perhaps in organelles, after incubation for 2 min. The intracellular 3OMG space after phloretin wash was slightly lower than that measured without wash. Separate experiments (not shown) demonstrated that the rate constant of efflux of labelled 3OMG after the addition of the stopping solution was less than 0.02 min^{-1}. Therefore, the decrease in 3OMG content after wash is probably due to a small loss of cells duering the procedure rather than efflux of 3OMG after the addition of phloretin. The distribution space for 3OMG after incubation for 2–3 min followed by the addition of stopping solution was measured in all transport experiments. The mean value was 15.5±2.6 μl per 10^8 cells (1 SD, $n=21$).

Fig.1A shows that 0.05 mM 3OMG equilibrates with a half-time of about 9 s. The inset shows a logarithmic transformation of the data with f_i being the fraction of the distribution space equilibrated at a given time. The almost linear relationship

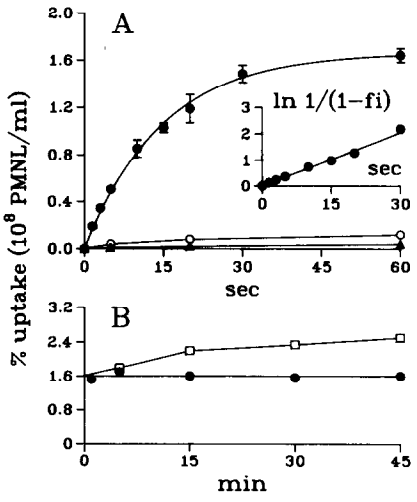

Fig.1. Time course of 3OMG and L-glucose uptake into PMNLs. The ordinate shows % uptake of ^{14}C-labelled 3OMG into PMNLs (10^8/ml). (A) Transport of 0.05 mM 3OMG in the absence (●—●) and presence (○—○) of 20 μM cytochalasin B. The distribution space for 3OMG was measured at 2 min in the absence of cytochalasin B as 16.4 μl/10^8 cells corresponding to 1.68% uptake into the cells. The inset shows a logarithmic transformation of the data and f_i is the fraction of the intracellular distribution space filled at a given time. The slope, i.e. the rate constant of entry *(v/S)*, is calculated by linear regression of the incubations for 0 to 10 s as 0.072 s^{-1}. Uptake of L-glucose is also shown (▲—▲). The points represent the mean of 3 values ± 1 SD when this exceeds the size of the symbol. (B) Phosphorylation of 0.05 mM 3OMG. Total radioactivity (□—□). Radioactivity which passed through an anion-exchange column (●—●), i.e. free 3OMG.

shows that entry follows an exponential course with a good approximation. The rate constant of entry (slope of the inset curve) is 0.072 s^{-1}. The mean value from this and 4 similar experiments was 0.080 ± 0.011 s^{-1} (1 SD). Since one cell contains about 16×10^{-8} μl (160 μm^3) water available for equilibration with 3OMG, this implies that about 13 μm^3 is initially equilibrated per s. The permeability is calculated from this value and the surface area of 243 μm^2 as 5.3×10^{-6} cm · s^{-1}. The permeability is of the same magnitude as that obtained in cultured human lymphocytes [7] and insulin-treated rat adipocytes [5] under comparable conditions, i.e. when using a very low 3OMG concentration. Fig.1A also shows that 20 μM cytochalasin B almost completely blocked the entry of 3OMG. Moreover, entry of L-glucose was negligible. The data show that 3OMG is rapidly transported across the PMNL membrane via a specific mechanism and that nonmediated diffusion plays only a negligible role.

Fig. 1B shows a slow progressive increase in the cell-associated ^{14}C activity when PMNLs were incubated for prolonged times. Most of the intracellular radioactivity passed through an anion-exchange column and this fraction (i.e. free 3OMG) remained constant from 1 to 45 min. In addition, there was an increasing amount retained on the column. This was interpreted to be phosphorylated 3OMG in analogy with previous results in perfused rat heart [10]. The phenomenon was presumably not due to a contamination since it was also found when the labelled 3OMG was purified by paper chromatography. In addition, no apparent phosphorylation was found in parallel experiments using rat hepatocytes or adipocytes (not shown). It should be noted that the apparent rate of phosphorylation is less than 0.5% of the rate of transport. Therefore, it does not interfere significantly with the calculation of initial transport velocities from incubations lasting only for seconds.

Fig.2 shows that labelled 3OMG can be transported against its concentration gradient, i.e. that f_i can transiently exceed unity when the concentration of unlabelled 3OMG is high in the intracellular water and low in the extracellular. Likewise, ^{14}C-labelled 3OMG was transported out of preequilibrated cells when unlabelled 3OMG was added to the extracellular buffer (not shown).

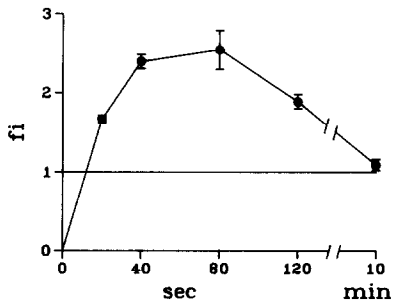

Fig.2. 3OMG countertransport. PMNLs were preincubated with 20 mM unlabelled 3OMG for 30 min. The cells were washed to remove unlabelled sugar analogue from the extracellular buffer and the ^{14}C-labelled 3OMG was added immediately after the wash. The horizontal line (f_i = 1) shows the intracellular distribution space for 0.05 mM 3OMG determined separately in cells preincubated in the absence of unlabelled 3OMG. Mean of 4 replicates ± 1 SD.

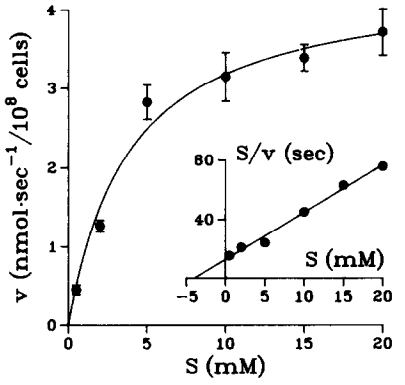

Fig.3. Concentration dependence of 3OMG exchange. PMNLs (1×10^8/ml) were equilibrated with unlabelled 3OMG as indicated for 15 min followed by incubations with labelled 3OMG for 4–12 s to achieve uptake of the tracer corresponding to about 30% of the distribution space (f_i about 0.30). The initial velocity (v) was calculated from the uptake at the given time according to the exponential relationship shown in fig.1A. The points are mean values of 4 replicates ± 1 SD. The inset shows the data plotted according to the Hanes' form of the Michaelis-Menten equation:

$$S/V = S/V_{max} + K_m/V_{max}$$

K_m is read from the intercept with the abscissa as 3.5 mM and V_{max} from the reciprocal slope as 0.30 mM · s^{-1}.

Thus, 3OMG exhibits countertransport in PMNL confirming that this sugar analogue is taken up by a specific and saturable transporter.

Fig.3 shows the concentration dependence of 3OMG equilibrium exchange entry, i.e. the sugar concentration is equal on both sides of the membrane and the labelled sugar is on the outside. In this situation the entry of the labelled sugar will follow an exponential course at any sugar concentration [5] and the initial velocities *(v)* are therefore readily calculated. It appears that v reaches a limiting value and the exchange is therefore clearly a saturable process. The inset shows that the data can be adequately described using Hanes' form of the Michaelis-Menten equation. The K_m of this experiment was 3.5 mM and V_{max} was 0.30 $mM \cdot s^{-1}$. The mean values of this and 4 similar experiments were with 1 SD: $K_m = 4.1 \pm 1.1$ mM; $V_{max} = 0.33 \times 0.99$ $mM \cdot s^{-1}$.

4. DISCUSSION

This report describes the first results on measurements of hexose transport in human PMNLs independent of metabolism. They show that 3OMG transport occurs via a specific and saturable transporter and that nonmediated diffusion is negligible. Following the transport, some 3OMG is metabolized and probably phosphorylated as shown in rat heart [10]. However, this process is so slow that it does not interfere with 3OMG entry measurements to any significant extent.

Previous authors may have measured 3OMG phosphorylation when transport was thought to be measured. This view is supported by the very low apparent 3OMG transport rates reported by Leroux et al. [1]. Thus, 10^{10} cells took up 2–3 nmol per min from a 0.05 mM 3OMG suspension according to fig.2 of [1]. It is seen from fig.1A that the initial velocity of transport using 0.05 mM 3OMG is in fact about 400 nmol per min by this amount of cells. A rate of 2–3 nmol per min is more in agreement with our rate of phosphorylation. It is likely that 3OMG transported into the cells in the previous study [1] was lost during washing of the cells (except the small amount that was phosphorylated) since a stopping solution was not employed. We believe, therefore, that 3OMG until now has been thought to be taken up by nonmediated diffusion in PMNLs [1,4] because the available methodology has not allowed the measurements of the rapid transport rates.

The kinetic constants reported here for 3OMG equilibrium exchange are quite different from the previously measured values using 30–60 min uptakes of 2-deoxyglucose. Thus, McCall et al. [3] found a K_m for deoxyglucose uptake of 0.5 mM (0.1 mM in cells stimulated by activated complement) as compared with our value of about 4 mM which is indistinguishable from the K_m for 3OMG exchange in adipocytes [11]. The V_{max} for 2-deoxyglucose uptake was reported as 30 nmol/min per 10^8 PMNLs [3] as compared with our 0.33 $mM \cdot s^{-1}$. The latter value is equivalent to 20 mmol/min per l 3OMG distribution space and, since 10^8 PMNLs possess 16 μl distribution space, 320 nmol/min per 10^8 PMNLs. Thus, our K_m and V_{max} values for 3OMG exchange are both one order of magnitude higher than those previously reported for 2-deoxyglucose uptake.

The reason for these differences may be one or more of the following possibilities. Firstly, 3OMG and 2-deoxyglucose may in fact exhibit widely different transport kinetics in PMNLs, although it seems unlikely because the 2 sugars have similar K_m values in the rat adipocyte [9]. Secondly, the PMNL hexose transporter may exhibit lower K_m and V_{max} values for transport into cells without sugar in the cytoplasmic phase (zero-trans entry) than for equilibrium exchange. Such asymmetry of the transport system has been reported in human erythrocytes [12] and lymphocytes [7]. Thirdly, the kinetic constants for 2-deoxyglucose uptake may reflect the rate of phosphorylation of this sugar more than its rate of transport in analogy with the situation in adipocytes [9]. In any case, since transport can now be measured independently of metabolism, it will be possible to answer these questions and to assess whether the rate of transport or the rate of phosphorylation determines the rate of conversion of hexoses to metabolic products, e.g. 2-deoxyglucose to 2-deoxyglucose phosphate and glucose to glycogen.

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